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Identification and Characterization of Di- and Tripeptide Transporter DtpT of *Listeria monocytogenes* EGD-e

Jeroen A. Wouters,[1]†‡ Torsten Hain,[2]† Ajub Darji,[2] Eric Hüfner,[1,2] Henrike Wemekamp-Kamphuis,[1] Trinad Chakraborty,[2] and Tjakko Abee[1]*

Wageningen Centre for Food Sciences (WCFS) and Laboratory of Food Microbiology, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands,[1] and Institute of Medical Microbiology, University of Giessen, 35392 Giessen, Germany[2]

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***Listeria monocytogenes* is a gram-positive intracellular pathogen responsible for opportunistic infections in humans and animals. Here we identified and characterized the *dtpT* gene (lmo0555) of *L. monocytogenes* EGD-e, encoding the di- and tripeptide transporter, and assessed its role in growth under various environmental conditions as well as in the virulence of *L. monocytogenes*. Uptake of the dipeptide Pro-[^{14}C]Ala was mediated by the DtpT transporter and was abrogated in a Δ*dtpT* isogenic deletion mutant. The DtpT transporter was shown to be required for growth when the essential amino acids leucine and valine were supplied as peptides. The protective effect of glycine- and proline-containing peptides during growth in defined medium containing 3% NaCl was noted only in *L. monocytogenes* EGD-e, not in the Δ*dtpT* mutant strain, indicating that the DtpT transporter is involved in salt stress protection. Infection studies showed that DtpT contributes to pathogenesis in a mouse infection model but has no role in bacterial growth following infection of J774 macrophages. These studies reveal that DptT may contribute to the virulence of *L. monocytogenes*.**

Listeriosis is a food-borne disease, the causative agent of which, *Listeria monocytogenes*, is a ubiquitously occurring, nonspore forming, gram-positive bacterium capable of growth in diverse habitats ranging from the soil to infected vertebrate hosts. *L. monocytogenes* is often isolated from foods of plant or animal origin and has been one of the food-related pathogens of greatest concern in recent decades. Various foods, such as meat, milk and other dairy products, and vegetables contaminated with *L. monocytogenes*, have been associated with human listeriosis (7, 28). Unlike other common food-borne diseases, listeriosis is associated with a mortality rate of >20%. Listeriosis occurs primarily in high-risk groups, such as neonates, the elderly, pregnant women, and immunocompromised individuals. The infectious dose for humans remains unknown and differs depending on the immune status of the individual. Important aspects of the presence of *L. monocytogenes* in food products are its abilities to acquire the required nutritive compounds and to survive a variety of environmental stresses (1, 15, 24).

L. monocytogenes has a limited biosynthetic capacity as judged by its complex nutritional requirements. In addition, *Listeria* spp. are unable to hydrolyze proteins, and their growth depends on other proteolytic systems that allow degradation of food proteins, including indigenous proteinases and proteinases derived from other microorganisms. Verheul et al. (30) demonstrated that the growth of *L. monocytogenes* was enhanced by *Pseudomonas fragi* and *Bacillus cereus* in a medium containing casein as the sole source of nitrogen. Previously, it was shown by Premaratne et al. (19) that *L. monocytogenes* Scott A requires the addition of six amino acids (Leu, Ile, Arg, Met, Val, and Cys) in a minimal medium. For other *L. monocytogenes* strains it has recently been shown that the amino acids methionine and cysteine are required for growth in a minimal medium (27). The biosynthesis pathways for all amino acids could be identified after metabolic reconstruction in *L. monocytogenes* EGD-e (8), and it was suggested that the requirements for amino acids may be due to repression of some amino acid biosynthetic pathways under laboratory conditions. Also, differences in the requirement for amino acids in defined medium have been reported for various *L. monocytogenes* strains (27).

In the recently characterized genome sequence of *L. monocytogenes* EGD-e, a total of 331 genes encoding different transport proteins are found, accounting for 11.6% of the total predicted genes (8). This is a relatively large number compared to those for bacteria whose genome sequences have been characterized thus far, and it reflects the ability of *Listeria* spp. to colonize and grow in a broad range of ecosystems, such as food products and host environments. Verheul et al. (29) identified a di- and tripeptide transport system in *L. monocytogenes* Scott A that resembles a proton motive force-dependent carrier protein. This peptide permease has a broad substrate specificity and allows transport of a variety of di- and tripeptides. Upon internalization, the peptides can be hydrolyzed by peptidases supplying (essential) amino acids for growth. In addition to the di- and tripeptide transporter, Verheul et al. (30) identified an ATP-dependent oligopeptide transporter capable of transporting peptides with as many as 8 residues. Recently, Borezee et

* Corresponding author: Mailing address: Laboratory of Food Microbiology, Wageningen University, Wageningen Centre for Food Sciences (WCFS), Bomenweg 2, 6703 HD Wageningen, The Netherlands. Phone: 31-317-484981. Fax: 31-317-484978. E-mail: Tjakko.Abee@wur.nl.

† J.A.W. and T.H. contributed equally to this work.

‡ Present address: Department of Flavour, NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands.

TABLE 1. *L. monocytogenes* strains, plasmids, and primers used in this study

Listeria plasmid or strain	Genotype, description, or sequence[a]	Resistance marker	Reference or source
Plasmids			
pCR2.1-TOPO	Shuttle vector	Amp	Invitrogen
pCR-XL-TOPO	Shuttle vector	Kan	Invitrogen
pAUL-A	Shuttle vector, thermosensitive	Em	5
pAUL-A+Δ*dtpT*	Shuttle vector with flanked *dtpT* gene regions	Em	This study
pPL2	*L. monocytogenes* site-specific phage integration vector	Cm	12
pPL2::*dtpT*	Integration vector haboring the *dtpT* gene	Cm	This study
Strains			
DH10β	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL endA1 nupG*		Invitrogen
EGD-e	Virulent wild type, clinical isolate, serovar 1/2a		8
Δ*dtpT*	EGD-e with *dtpT* deleted		This study
Δ*dtpT* + *pPL2*::*dtpT*	Δ*dtpT* complemented with *dtpT*		This study
Primers			
A	CAAATTCACGCATTTCAGC		This study
B	GTGGGCGGCCGCTAGGAATTAAGCGCACTTTC		This study
C	GTGGGCGGCCGCTCCGTAATAGGAGAATCGC		This study
D	TGGAACCTTATCCAGAGG		This study
E	GTGGTAGACTTACTCGTT		This study
F	CCGATCAAGTGTGTTTAA		This study

[a] Underlined bases indicate NotI restriction sites.

al. (4) identified the genes encoding this transport system. The *opp* operon consists of five genes and shows characteristics typical of ABC transporters. Deletion of *oppA* revealed that it is indeed involved in the transport of oligopeptides and that it is required for growth at low temperatures. The OppA transporter is also involved in intracellular growth of *L. monocytogenes* in bone marrow-derived macrophages, but an *oppA* deletion mutant was only slightly less virulent than the wild type when examined in the mouse model of tissue and organ colonization (4).

L. monocytogenes is able to grow at salt concentrations as high as 10% (15) and at temperatures as low as 0°C (31). For the adaptation to both low temperatures and high salt conditions, the ability to accumulate compounds, so-called compatible solutes such as proline, ectoine, glycine, glutamate, carnitine, and glycine betaine, from the external environment is essential. The intracellular accumulation of these solutes allows bacterial survival at high or fluctuating osmolarities or under conditions of desiccation or freezing. This type of compound can be accumulated to high concentrations without affecting intracellular processes (reviewed by Sleator et al. [24]). Effective compatible solutes for *L. monocytogenes* are glycine betaine and carnitine (3), and several transport systems for these substances have been characterized in recent years (11, 22, 25, 32). In addition, it was shown that specific glycine- and proline-containing peptides stimulate growth of this bacterium at high osmotic strength. The peptide prolyl-hydroxylproline accumulates in cells to high levels in response to growth at high osmolarity, and the pools of the derived amino acids also show a dependence on the external osmotic pressure (2).

In this work we identify and characterize the di- and tripeptide transporter DtpT of *L. monocytogenes* and assess its role in growth under different conditions. Our data indicate that the DtpT transporter may contribute to the growth and survival of *L. monocytogenes* in the environment as well as in the infected host.

MATERIALS AND METHODS

DNA isolation and manipulations. The procedures for the isolation of plasmid and chromosomal DNA from *L. monocytogenes* have been described previously (21). Standard protocols were used for recombinant DNA techniques (20).

Bacterial strains, media, growth conditions, and chemicals. Bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* EGD-e was grown in brain heart infusion (BHI) broth (Difco) at 37°C. *Escherichia coli* was grown in Luria-Bertani broth (LB) (Difco) at 37°C. Ampicillin (100 μg/ml) and erythromycin (300 μg/ml for *E. coli*, 5 μg/ml for *L. monocytogenes*) were added to broth or agar as needed. *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* were also cultured in chemically defined medium (CDM) as described previously by Premaratne et al. (19). To analyze the ability to use di- and tripeptides, the essential amino acids leucine and valine were omitted from CDM and these amino acids were supplied as di- or tripeptides (Leu-Ala, Ala-Leu, Leu-Pro, Leu-Gly-Gly, Ala-Leu-Gly, Val-Gly, and Ala-Val). Growth was monitored by measuring the optical density at 600 nm (OD_{600}) (Novaspec II spectrophotometer; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The di- and tripeptides were obtained from Sigma Chemicals (St. Louis, MO). To assay the role of the DtpT transporter in salt stress and/or low-temperature stress, the growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* was also monitored in BHI containing NaCl at concentrations of 3, 6, and 9% (wt/vol) at several temperatures (37, 10, 7, and 2.5°C). The possible osmoprotective roles of the glycine- or proline-containing peptides Val-Gly and Leu-Pro were analyzed for both *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* in CDM containing 3% (wt/vol) NaCl (CDMS) plus 1 mM of Leu-Pro or Gly-Val. *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* were also grown in sterilized milk and meat bouillon as models for growth of *L. monocyt*ogenes on food products. Growth was measured as CFU.

Generation of the *L. monocytogenes* Δ*dtpT* deletion mutant. A *dtpT* chromosomal in-frame deletion mutant was constructed by deletion of a 1,383-bp internal fragment of the *dtpT* gene. In order to create the *dtpT* mutant, the flanking regions of the *dtpT* gene were amplified using PCR. The upstream region was generated by using primers A and B (Table 1), resulting in PCR amplification of a 415-bp fragment. The downstream region was synthesized with the primer pair C and D (Table 1) to obtain a fragment of 603 bp. Both PCR products harbored NotI restriction endonuclease sites, which were introduced with oligonucleotides B and C. Purified PCR products were digested with NotI and ligated. Following overnight ligation, a part of the ligation mix was added to an amplification with primers A and D for the flanking fragments to generate a PCR product of 1,002 bp harboring the 1,383-bp deletion of the *dtpT* gene. The PCR product was cloned into the shuttle cloning vector pCR2.1-TOPO. The cloned fragment was digested with restriction endonucleases BamHI and SalI and ligated directly to the temperature-sensitive suicide vector pAUL-A (5) digested with the same

restriction endonucleases, BamHI and SalI. Plasmid DNA of pAUL-A-Δ*dtpT* was isolated from recombinants and introduced into wild-type *L. monocytogenes* EGD-e as previously described by Schaferkordt and Chakraborty (21). The in-frame chromosomal deletion was finally confirmed by sequencing the PCR product generated with oligonucleotides A and D from chromosomal DNA of the *L. monocytogenes* Δ*dtpT* mutant.

Complementation of the *L. monocytogenes* Δ*dtpT* deletion mutant. pPL2, a site-specific phage integration vector (12), was used for complementation of the Δ*dtpT* mutant. The *dtpT* gene and flanking regions were amplified using oligonucleotides E and F (Table 1), generating a 1,876-bp fragment, which was introduced into the pCR-XL-Topo vector (Invitrogen). The ligation mixture was transformed by electroporation into *E. coli* DH10β and plated onto LB agar plates containing 50 μg/ml kanamycin. Plasmid DNA was isolated from a *dtpT*-harboring recombinant and digested with the restriction endonucleases BamHI and XhoI to release the inserted DNA. Following agarose gel electrophoresis, the *dtpT*–harboring fragment was isolated and ligated to BamHI/XhoI-restricted vector pPL2 DNA. Following electroporation of the ligation mixture, recombinant *E. coli* DH10β clones were plated onto LB agar plates containing 25 μg/ml of chloramphenicol. One representative recombinant, pPL2::*dtpT*, was sequenced to verify the authenticity of the gene cloned and introduced into the isogenic Δ*dtpT* mutant strain by electroporation. Selection of transformants was performed on BHI plates supplemented with 7.5 μg/ml of chloramphenicol. Insertion of the pPL2::*dtpT* plasmid into the PSA bacteriophage attachment site at $tRNA^{Arg}$-*attBB′* was verified by using the primer pair NC16 and PL95 (Table 1) to specifically amplify a 499-bp PCR product in the integrant strains. The primer pair E and F was used to specifically detect the *dtpT* gene region. The complemented strain was examined for further phenotypic analysis.

Transport studies. *L. monocytogenes* EGD-e, *L. monocytogenes* Δ*dtpT*, and *L. monocytogenes* Δ*dtpT* complemented with *dtpT* were grown in BHI to mid-exponential phase (OD_{600}, 0.5). Cells were harvested by centrifugation (3,000 × *g*, 15 min, 10°C), washed twice, and resuspended in 240 mM sodium PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.0) containing 5 mM $MgSO_4$ and 100 μg chloramphenicol per ml (and containing 3% NaCl when cells were grown in the presence of an additional 3% NaCl). Cells at an OD_{600} of 2 were 10-fold concentrated in this buffer and stored on ice until use. Cells (final OD_{600}, 1) were preenergized at 37°C with 10 mM glucose for 10 min prior to the addition of radiolabeled proline-[^{14}C]alanine (final concentration, 6.5 μM). Pro-[^{14}C]Ala was a generous gift from Gang Fang and Bert Poolman (University of Groningen, Groningen, The Netherlands) (6) and was prepared as described by Hagting et al. (10). Samples were withdrawn, and uptake was stopped by the addition of 2 ml of 240 mM sodium PIPES buffer (pH 6.0) containing 5 mM $MgSO_4$ (and containing 3% NaCl when uptake experiments were performed in the presence of NaCl). The cells were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under a vacuum. The filters were washed with another 2 ml of 50 mM potassium phosphate (pH 6.8) buffer, and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 1600 TR; Packard Instruments Co., Downers Grove, IL). Uptake of Pro-[^{14}C]Ala was normalized to the total quantity of cellular proteins, which was determined by using the bicinchoninic acid method as provided by the supplier (Sigma Chemicals, St. Louis, MO) with bovine serum albumin as a standard.

Intracellular infection assay. The murine macrophage-like cell line J774.A1 (ATCC TIB-67) was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Cells were incubated in the presence of 5% CO_2 at 37°C. Infection assays were performed as described previously (13) using bacteria at a multiplicity of infection between 1 and 20. After 1 h of incubation, the tissue culture plates were washed twice with phosphate-buffered saline before the addition of cell culture medium containing 100 μg/ml of gentamicin. After 2, 4, and 8 h, the monolayer was lysed with 0.1% Triton X-100. The number of bacteria released was expressed in CFU per milliliter after appropriate dilutions were plated onto BHI agar plates. Experiments were carried out three times with three replicates each.

Mouse virulence assay. Six- to eight-week-old female BALB/c mice (obtained from Harlan Winkelmann, Germany) were used in experiments to study the effect of deletion of *dtpT* on the virulence of *L. monocytogenes*. For the in vivo bacterial infection and the survival and growth analyses, groups of five mice were infected in vitro with *L. monocytogenes* EGD-e, mutant *L. monocytogenes* Δ*dtpT*, and the complemented Δ*dtpT*::*dtpT* strain. The kinetics of bacterial growth in the spleen and liver were monitored. For this purpose, fresh bacterial cultures were established in BHI medium from overnight cultures. After 2 to 3 h of incubation at 37°C, the cells were harvested by centrifugation and subsequently washed and resuspended in saline solution. Mice were infected in vivo in the tail vein with 200 μl saline containing 1,000 to 1,500 bacteria. All data presented for the infection studies with mice are representative of at least three independent experiments. For statistical analysis, the significance of these data was calculated using a paired Student *t* test. Data are expressed as means ± standard deviations.

RESULTS

Identification of the *dtpT* gene encoding a putative di- and tripeptide transporter. Based on sequence homology and motif searches, a *dtpT* homolog (lmo0555) was identified in the *L. monocytogenes* EGD-e genome sequence (8). The 1,479-bp gene encodes a single protein of 492 amino acid residues. The listerial DtpT protein is similar to the eukaryotic proton motive force-dependent peptide transporter (PTR) family (26) and several bacterial DtpT systems. The *dtpT* gene shows 53 and 48% identity to the *yclF* gene of *Bacillus subtilis* (33) and the *dtpT* gene of *Lactococcus lactis* (9), respectively. Bacterial DtpT transporter proteins have been shown to be responsible for the transport of hydrophilic di- and tripeptides and to share similarities with eukaryotic peptide transporters, e.g., from yeasts, plants, and the kidneys and small intestines of rabbits, humans, and rats. Based on homology and the hydropathy profile, it can be predicted that the DtpT protein consists of a bundle of 12 transmembrane α-helices with the N and C termini located internally. The PTR signature motif FSIFYMGINLGAF, present between amino acid residues 150 and 162, is located within the fifth putative membrane segment of the DptT polypeptide.

The *dtpT* gene of *L. monocytogenes* EGD-e is preceded by a putative SigA-regulated promoter, suggesting that this gene is expressed under normal growth conditions. Thirteen base pairs downstream of the open reading frame encoding DtpT, a putative rho-independent terminator sequence could be identified. These data would suggest a monocistronic transcript for the *dtpT* gene. The genes surrounding *dtpT* do not seem to be involved in processes related to the transport and/or utilization of di- and tripeptides. Upstream of *dtpT*, a gene encoding an NADH-dependent butanol dehydrogenase is present, while a putative operon comprising two genes encoding phosphoglycerate mutases is located downstream of *dtpT*. All of these genes have separate terminators and do not seem to share common genetic elements of regulation with *dtp*T (Fig. 1).

Transport of Pro-[^{14}C]Ala. Transport of the dipeptide Pro-[^{14}C]Ala in wild-type *L. monocytogenes*, *L. monocytogenes* Δ*dtpT*, and *L. monocytogenes* Δ*dtpT* complemented with a chromosomally encoded *dtpT* gene was analyzed. In *L. monocytogenes* EGD-e, significant Pro-[^{14}C]Ala transport is observed for mid-exponential cells grown in defined medium (Fig. 2). These levels are comparable to the uptake rates for *L. monocytogenes* Scott A reported by Verheul et al. (29). Next, the transport of Pro-[^{14}C]Ala was analyzed in *L. monocytogenes* Δ*dtpT*; for this strain, no transport activity could be measured (Fig. 2). Complementation of the *dtpT* gene in *L. monocytogenes* Δ*dtpT* revealed that dipeptide transport was restored to levels similar to that observed for *L. monocytogenes* EGD-e. These data clearly demonstrate the role of DtpT in the transport of the Pro-[^{14}C]Ala dipeptide. Previously, it was shown that the transport of dipeptides is dependent on the proton motive force. Addition of the potassium ionophore valinomycin and the potassium proton exchanger nigericin sig-

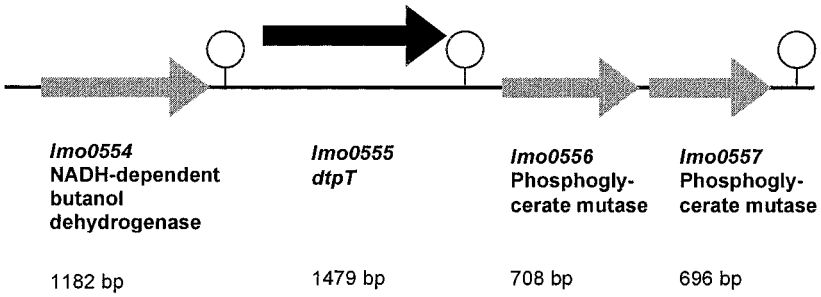

FIG. 1. Organization of *dtpT* (black arrow) and surrounding genes (gray arrows) in *L. monocytogenes* EGD-e. The hairpin structures indicate putative terminators. The numbering of the open reading frames follows the numbering of the *L. monocytogenes* EGD-e genome sequence (8).

nificantly inhibited this transport (29). Here we demonstrate that the transport of Pro-[^{14}C]Ala can be attributed to the DtpT transporter.

The ability of *L. monocytogenes* Δ*dtpT* to grow on di- and tripeptides is affected. The growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* on a defined medium containing different peptides was monitored. For this purpose, di- and tripeptides containing the essential amino acid valine or leucine were added to CDM from which either valine or leucine was omitted, respectively. In the absence of either valine or leucine in CDM, no growth of *L. monocytogenes* EGD-e at 37°C was observed within 60 h (Fig. 3), indicating the requirement of both these amino acids for *L. monocytogenes* EGD-e. Upon addition of the leucine-containing peptide Leu-Ala, Ala-Leu, Leu-Pro, Leu-Gly-Gly, or Ala-Leu-Gly, growth was completely restored (Fig. 3A), indicating the efficient uptake and probable subsequent hydrolysis of these peptides and their usage for growth by *L. monocytogenes* EGD-e.

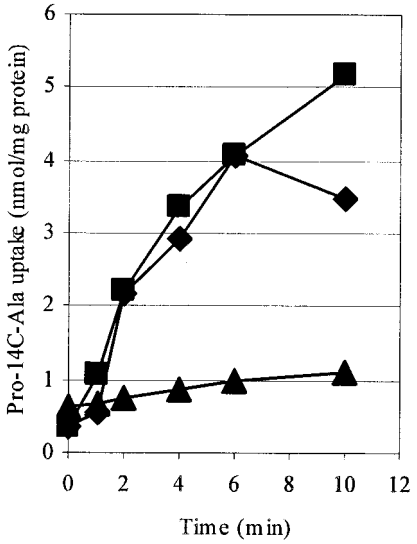

FIG. 2. Uptake of the Pro-[^{14}C]Ala peptide in *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT*. Cells were grown in BHI to mid-exponential phase (OD_{600}, 0.5) and washed twice in 240 mM sodium PIPES buffer (pH 6.0) containing 5 mM $MgSO_4$. Assays of Pro-[^{14}C]Ala uptake were performed in 240 mM sodium PIPES buffer (pH 6.0) containing 5 mM $MgSO_4$ with glucose-energized cells of *L. monocytogenes* EGD-e (squares), *L. monocytogenes* Δ*dtpT* (triangles), or *L. monocytogenes* Δ*dtpT* complemented with *dtpT* (diamonds).

The addition of the dipeptide Val-Gly completely restored growth of *L. monocytogenes* EGD-e, whereas the addition of Ala-Val did not result in restoration of growth under these conditions (Fig. 3C). In the case of *L. monocytogenes* Δ*dtpT*, growth on CDM lacking leucine could not be restored by the addition of leucine-containing dipeptides, but after an extended lag phase, growth was observed in the presence of the tripeptide Leu-Gly-Gly or Ala-Leu-Gly (Fig. 3B). Also, *L. monocytogenes* Δ*dtpT* did not grow in CDM lacking valine and supplemented with the valine-containing dipeptide Val-Gly, indicating the inability to use this substrate for growth (Fig. 3D). These data indicate that deletion of DtpT results in the inability of *L. monocytogenes* to use leucine- or valine-containing dipeptides for growth.

Growth of *L. monocytogenes* Δ*dtpT* in BHI, in minimal medium at high salt concentrations, and in food model systems. The growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* was also monitored in BHI medium, and this revealed no differences in growth for these strains at 37°C. Similarly, in two food products, sterilized milk and beef bouillon, no differences in growth at 37°C were observed for *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT*, as measured by the number of CFU. Deletion of *dtpT* also did not alter the ability of *L. monocytogenes* to grow in BHI at low temperatures (10°C, 7°C, or 2.5°C) without or with added salt (up to 10% NaCl) (data not shown). Probably, growth of *L. monocytogenes* in BHI does not depend on the use of di- or tripeptides for a supply of amino acids. Previously, a protective effect of glycine- and/or proline-containing peptides was reported for *L. monocytogenes* ATCC 23074 (2). To further investigate this, we added a glycine- or a proline-containing dipeptide (final concentration, 1 mM) to CDMS, which contains all essential amino acids. This demonstrated that for *L. monocytogenes* EGD-e, growth at 37°C at high salt concentrations was stimulated in the presence of the peptides, whereas no stimulation was observed for *L. monocytogenes* Δ*dtpT*. This implies that the DtpT transporter is involved in the transport of peptides and that these peptides, or the corresponding amino acids, can counter high-osmolarity stress in *L. monocytogenes* EGD-e (Fig. 4).

DtpT contributes to the pathogenesis of *L. monocytogenes* in mice. Infection studies were performed in vitro using J774A.1 macrophages. *L. monocytogenes* EGD-e, the Δ*dtpT* mutant, and the chromosomally complemented Δ*dtpT*::*dtpT* strain were used to infect monolayers of J774A.1 cells. No discernible differences were found between the abilities of the

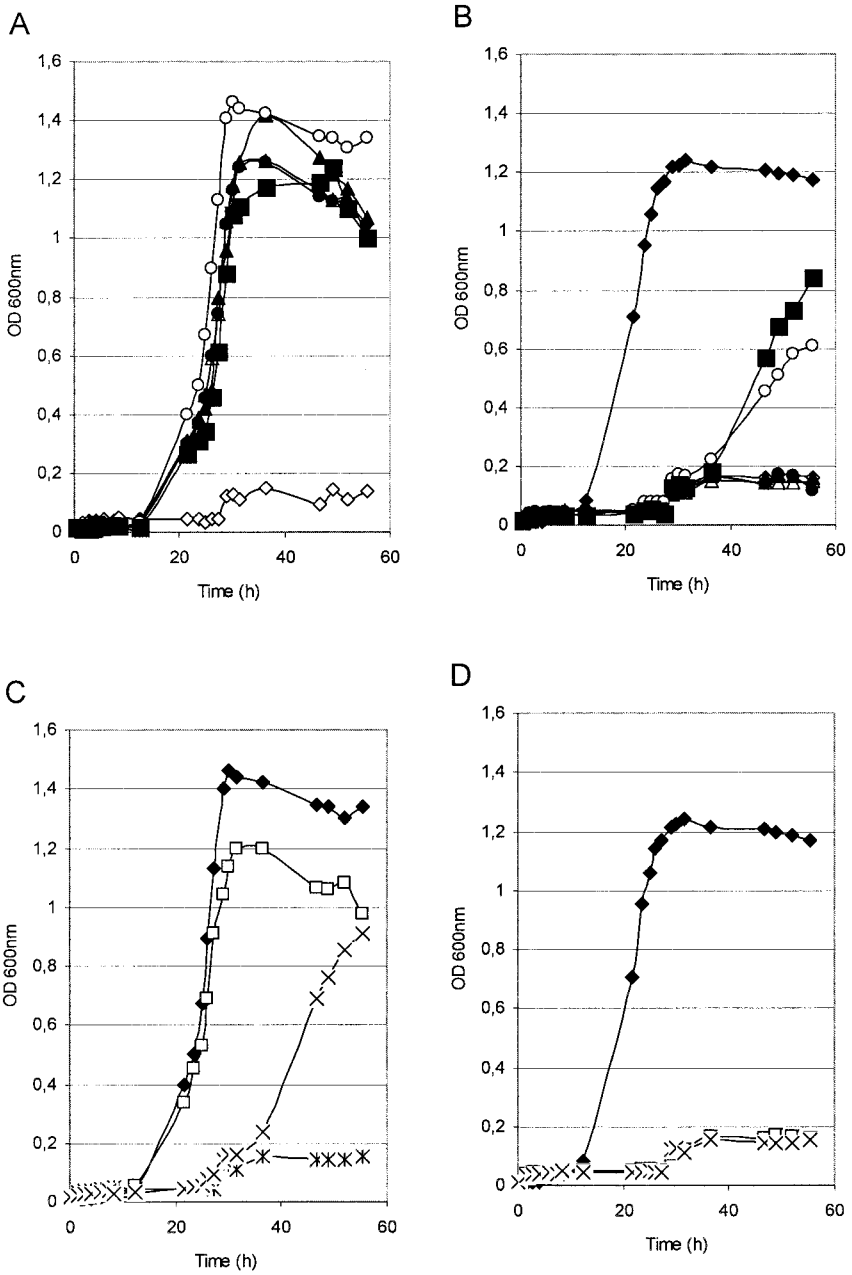

FIG. 3. Valine- or leucine-containing peptides as sources of essential amino acids for growth of *L. monocytogenes*. The strains were cultured at 37°C in CDM (closed diamonds) and in CDM lacking leucine (open diamonds) or lacking valine (starbursts). *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* were also cultured in CDM lacking leucine and complemented with Leu-Ala (closed triangles), Ala-Leu (open triangles), Leu-Pro (closed circles), Leu-Gly-Gly (open circles), or Ala-Leu-Gly (closed squares), whereas CDM lacking valine was complemented with Val-Gly (open squares) or Ala-Val (multiplication signs). Growth was monitored spectrophotometrically at OD_{600}. (A and B) Growth of *L. monocytogenes* EGD-e (A) or *L. monocytogenes* Δ*dtpT* (B) in CDM, CDM lacking leucine, and CDM lacking leucine but supplied with the leucine-containing peptide Leu-Ala, Ala-Leu, Leu-Pro, Leu-Gly-Gly, or Ala-Leu-Gly. (C and D) Growth of *L. monocytogenes* EGD-e (C) and *L. monocytogenes* Δ*dtpT* (D) in CDM, CDM lacking valine, and CDM lacking valine but supplied with the peptide Val-Gly or Ala-Val.

wild-type and mutant strains to proliferate intracellularly (Fig. 5). By using a mouse infection model, studies were performed comparing the virulence properties of *L. monocytogenes* Δ*dtpT* and the complemented Δ*dtpT*::*dtpT* strain to that of *L. monocytogenes* EGD-e. Although similar inocula were used for all three bacteria for in vivo growth kinetics, we found significantly lower numbers of Δ*dtpT* mutant bacteria than of complemented or wild-type EGD-e bacteria in both the spleens and livers (Fig. 6A and B, respectively) of infected mice starting from day 2 postinfection ($P < 0.05$). This effect was most pronounced after day 3 and day 4 postinfection. These results indicate an impairment in the survival and/or growth of the *L. monocytogenes* Δ*dtpT* strain in these organs in vivo.

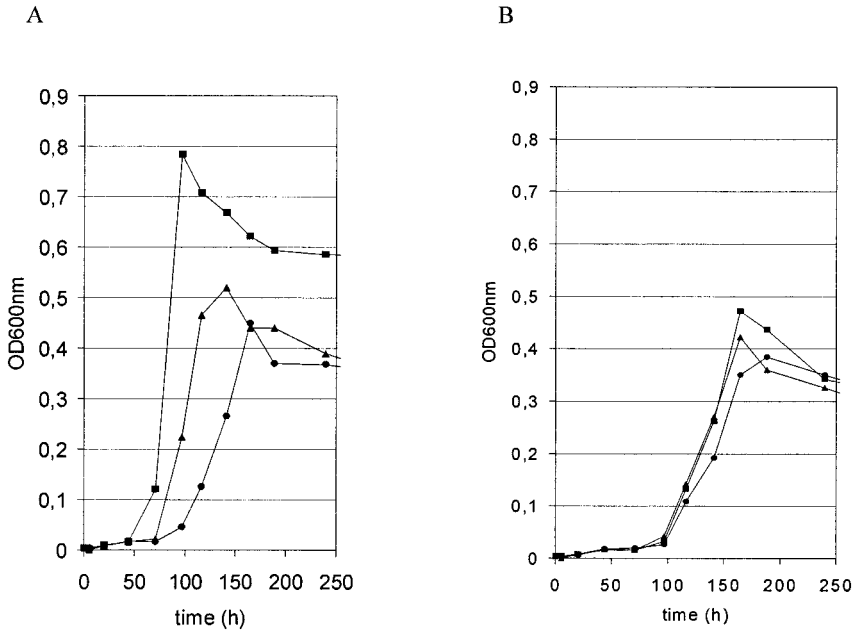

FIG. 4. Growth of *L. monocytogenes* EGD-e (A) and *L. monocytogenes* Δ*dptT* (B) in CDMS containing a glycine- or proline-containing peptide. Growth was monitored at 37°C by measuring the OD_{600}. Circles, growth in CDMS; squares, growth in CDMS containing 1 mM Leu-Pro; triangles, growth in CDMS containing 1 mM Gly-Val.

DISCUSSION

In this report, we demonstrate that peptides are nutritionally valuable compounds providing *L. monocytogenes* with essential amino acids. Using a Δ*dptT* strain, we show that the di- and tripeptide transporter DtpT is involved in the translocation of various di- and tripeptides. Transport of the Pro-[^{14}C]Ala dipeptide was abolished in a *dtpT* deletion mutant and restored in a complementation mutant. Growth experiments with CDM demonstrated that DtpT is responsible for the uptake of leucine- and valine-containing di- and tripeptides that can be sources of the essential amino acids leucine and valine. Previously, it was demonstrated that *L. monocytogenes* has intracellular aminopeptidase activity responsible for the degradation of the peptides, thus generating a pool of free amino acids (29). In the presence of the peptide Leu-Gly-Gly or Ala-Leu-Gly, growth of the Δ*dtpT* mutant is observed only after extended periods of incubation. This phenomenon is most likely explained by the presence of another transport system capable of translocating these peptides. The oligopeptide transporter Opp, previously physiologically characterized by Verheul et al. (30) and recently genetically characterized by Borezee et al. (4), is the most likely candidate for this transport activity. It has been demonstrated that Opp is capable of transporting peptides longer than 4 residues. It is conceivable that Opp is also capable of transporting tripeptides at low rates.

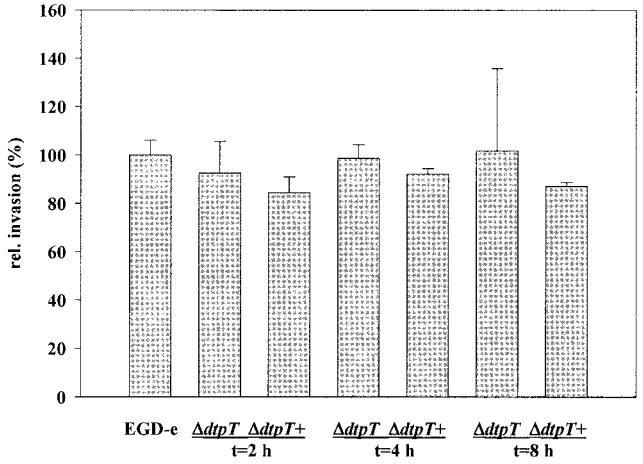

FIG. 5. Infection studies of the wild-type, Δ*dtpT*, and Δ*dtpT*+pPL2::*dtpT* strains. The effect of *dtpT* deletion on the intracellular replication of *L. monocytogenes* in the murine macrophage-like cell line J774A.1 was investigated. Cell monolayers were infected with approximately 1 bacterium per cell. After 1 h of incubation, cells were incubated for 1, 3, and 7 h in the presence of gentamicin (2-, 4-, and 8-h time points). Bacterial numbers, determined for the Δ*dtpT* and Δ*dtpT*+pPL2::*dtpT* strains at the indicated time points, were compared to the numbers of EGD-e bacteria (set at 100% invasion at each time point) to obtain relative percentages of invasion (*y* axis).

L. monocytogenes is a bacterium that is able to adapt to a wide variety of stressful conditions, and various defensive mechanisms have been reported to be involved in these adaptation processes (1). For certain proline- and glycine-containing peptides, a protective effect at high osmolarity has been reported (2) Here we demonstrate that in BHI medium, milk, or beef bouillon, the ability to grow is not dependent on DtpT, due to the availability of free amino acids. Also when *L. monocytogenes* is grown in BHI at low temperatures without or with added NaCl (up to 10% [wt/vol]), loss of DtpT transporter activity does not significantly affect growth. In a more controlled setting using CDMS, a growth-stimulatory effect of peptides containing proline (Leu-Pro) or glycine (Val-Gly) was found for the wild type but not the Δ*dtpT* mutant. This indi-

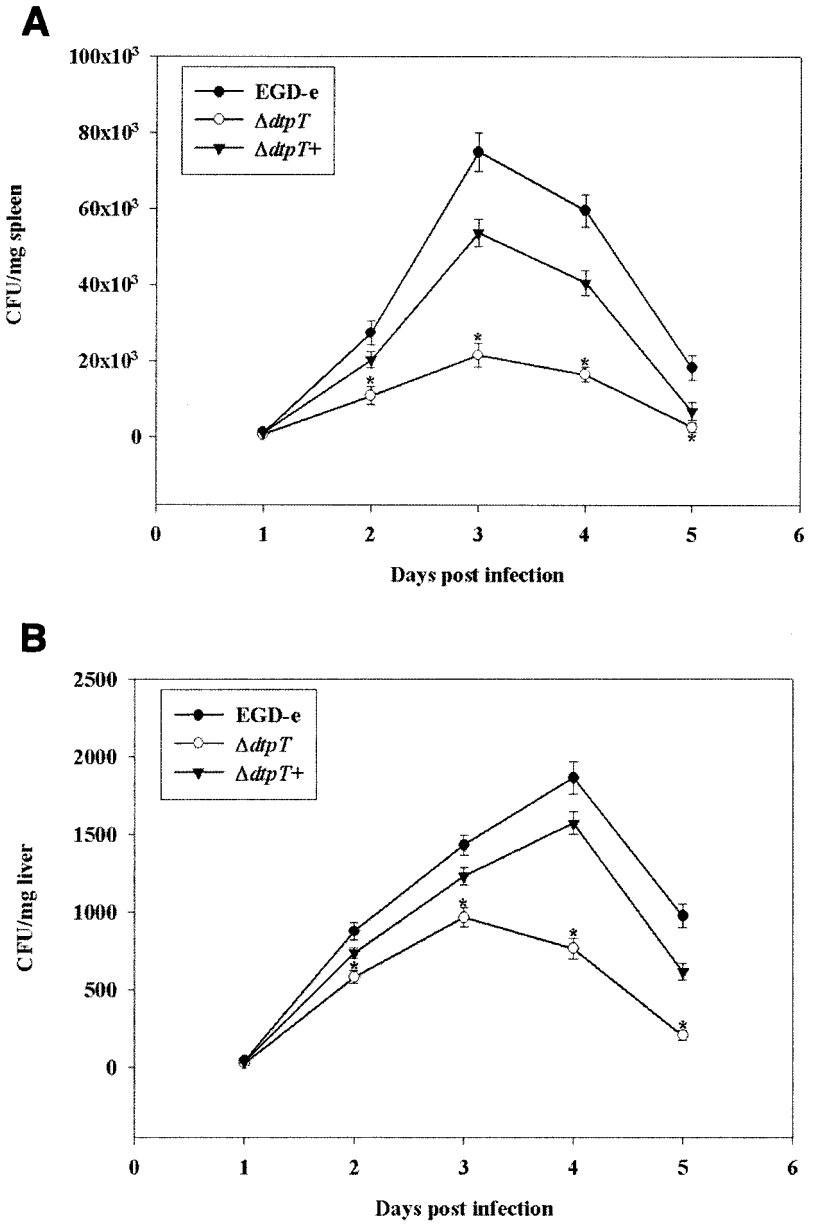

FIG. 6. Virulence of *L. monocytogenes* EGD-e, *L. monocytogenes* Δ*dtpT*, and *L. monocytogenes* Δ*dtpT*+pPL2::*dtpT* in a mouse model. Mice were infected in vivo with approximately 10^3 bacteria. Bacteria from the spleen (A) and the liver (B) were enumerated at days 1, 2, 3, 4, and 5 postinfection. Error bars represent the standard deviations from the means ($n = 3$). Asterisks indicate that the means are significantly different from those for the wild type ($P < 0.05$).

cates that DtpT may contribute to stress resistance under specific conditions when protective di- or tripeptides can be taken up from the environment.

A role for genes encoding proteins involved in stress tolerance and/or amino acid metabolism of *L. monocytogenes* has been reported previously (11, 14, 22, 23, 24, 32). Here we provide evidence for a role of DtpT in the listerial virulence pathway. It is demonstrated that DtpT contributes to growth and survival of the bacterium in the mouse model of infection. However, we observed that the DtpT transporter was not required for invasion and multiplication in the mouse macrophage-like cell line J774A.1. During systemic infection of mice, many compartments are breached and the bacterium encounters a variety of microenvironments. Uptake of dipeptides through the DtpT transporter may contribute to survival in some of these environments, a condition not represented by use of the J774A.1 cell line. In low-GC gram-positive organisms, di- and tripeptide transporters are thought to play an important role in regulation or signaling processes. In *B. subtilis* and *L. lactis*, the levels of branched-chain amino acids (isoleucine, leucine, and valine) trigger the central effector CodY, a pleiotropic transcription regulator that has broad effects on gene expression (17, 18). The reduced capacity of the Δ*dtpT* mutant to grow and survive in the mouse infection

model may also be indirect, resulting from breakdown of signaling mechanisms mediated by the listerial CodY gene product. Additional studies will be needed to confirm this hypothesis.

Recently a study on PrfA, the major regulator of listerial virulence gene expression (5), indicated that this protein can act as an activator and as a repressor of different sets of genes, depending on the growth conditions used (16). One of the genes activated by PrfA is lmo0555, i.e., the *dtpT* gene. Although this gene does not have a putative PrfA box, transcription of this gene in BHI at 37°C was lower in a strain lacking PrfA (16). The exact regulation of *dtpT* transcription in *L. monocytogenes* remains to be elucidated.

The results of this study contribute to our understanding of the utilization of peptides by *L. monocytogenes*. We have shown that DtpT is responsible for the translocation of di- and tripeptides, contributes to stress resistance under specific conditions, and may contribute to the virulence of *L. monocytogenes*.

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REFERENCES

1. **Abee, T., and J. A. Wouters.** 1999. Microbial stress in minimal processing. Int. J. Food Microbiol. **50:**65–91.
2. **Amezaga, M. R., I. Davidson, D. McLaggan, A. Verheul, T. Abee, and I. R. Booth.** 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology **141:**41–49.
3. **Beumer, R. R., M. C. te Giffel, L. J. Cox, F. M. Rombouts, and T. Abee.** 1994. Effect of exogenous proline, betaine, and carnitine on growth of *Listeria monocytogenes* in a minimal medium. Appl. Environ. Microbiol. **60:**1359–1363.
4. **Borezee, E., E. Pellegrini, and P. Berche.** 2000. OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. Infect. Immun. **68:**7069–7077.
5. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
6. **Fang, G., W. N. Konings, and B. Poolman.** 2000. Kinetics and substrate specificity of membrane-reconstituted peptide transporter DtpT of *Lactococcus lactis*. J. Bacteriol. **182:**2530–2535.
7. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
8. **Glaser, P., L. Frangeul, C. Buchreiser, C. Rusniok, et al.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
9. **Hagting, A., E. R. S. Kunji, K. J. Leenhouts, B. Poolman, and W. N. Konings.** 1994. The di- and tripeptide transport protein of *Lactococcus lactis*. J. Biol. Chem. **269:**11391–11399.
10. **Hagting, A., J. Knol, B. Hasemeier, M. R. Streutker, G. Fang, B. Poolman, and W. N. Konings.** 1997. Amplified expression, purification and functional reconstitution of the dipeptide and tripeptide transport protein of *Lactococcus lactis*. Eur. J. Biochem. **247:**581–587.
11. **Ko, R., and L. T. Smith.** 1999. Identification of an ATP-driven, osmoregulated glycine betaine transport system in *Listeria monocytogenes*. Appl. Environ. Microbiol. **65:**4040–4048.
12. **Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar.** 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J. Bacteriol. **184:**4177–4186.
13. **Lingnau, A., E. Domann, M. Hudel, M. Bock, T. Nichterlein, J. Wehland, and T. Chakraborty.** 1995. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. Infect. Immun. **63:**3896–3903.
14. **Marquis, H., H. G. Bouwer, D. J. Hinrichs, and D. A. Portnoy.** 1993. Intracytoplasmic growth and virulence of *Listeria monocytogenes* auxotrophic mutants. Infect. Immun. **61:**3756–3760.
15. **McClure, P. J., T. M. Kelly, and T. A. Roberts.** 1991. The effects of temperature, pH, sodium chloride and sodium nitrate on growth of *Listeria monocytogenes*. Int. J. Food Microbiol. **14:**77–92.
16. **Milohanic, E., P. Glaser, J.-Y. Coppée, L. Frangeul, Y. Vega, J. A. Vázquez-Boland, F. Kunst, P. Cossart, and C. Buchreiser.** 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol. Microbiol. **47:**1613–1625.
17. **Molle, V., Y. Nakaura, R. P. Shivers, H. Yamaguchi, R. Losick, Y. Fujita, and A. L. Sonenshein.** 2003. Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. J. Bacteriol. **185:**1911–1922.
18. **Petranovic, D., E. Guedon, B. Sperandio, C. Delorme, D. Ehrlich, and P. Renault.** 2004. Intracellular effectors regulating the activity of the *Lactococcus lactis* CodY pleiotropic transcription regulator. Mol. Microbiol. **53:**613–621.
19. **Premaratne, R. J., W. J. Lin, and E. A. Johnson.** 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57:**3046–3048.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Schaferkordt, S., and T. Chakraborty.** 1995. Vector plasmid for insertional mutagenesis and directional cloning in *Listeria* spp. BioTechniques **19:**720–725.
22. **Sleator, R. D., C. G. M. Gahan, T. Abee, and C. Hill.** 1999. Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28. Appl. Environ. Microbiol. **65:**2078–2083.
23. **Sleator, R. D., C. G. M. Gahan, and C. Hill.** 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. Appl. Environ. Microbiol. **67:**2571–2577.
24. **Sleator, R. D., C. G. M. Gahan, and C. Hill.** 2003. A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*. Appl. Environ. Microbiol. **69:**1–9.
25. **Sleator, R. D., J. Wouters, C. G. M. Gahan, T. Abee, and C. Hill.** 2001. Analysis of the role of OpuC, an osmolyte transporter system, in salt tolerance and virulence potential of *Listeria monocytogenes*. Appl. Environ. Microbiol. **67:**2692–2698.
26. **Steiner, H. Y., F. Naider, and J. M. Becker.** 1995. The PTR family: a new group of peptide transporters. Mol. Microbiol. **16:**825–834.
27. **Tsai, H. N., and D. A. Hodgson.** 2003. Development of a synthetic minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **69:**6943–6945.
28. **Vázquez-Boland, J. A., M. Kuhn, P. Berghe, T. Chakraborty, G. Domínquez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.
29. **Verheul, A., A. Hagting, M.-R. Amezaga, I. R. Booth, F. M. Rombouts, and T. Abee.** 1995. A di- and tripeptide transport system can supply *Listeria monocytogenes* Scott A with amino acids essential for growth. Appl. Environ. Microbiol. **61:**226–233.
30. **Verheul, A., F. M. Rombouts, and T. Abee.** 1998. Utilization of oligopeptides by *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. **64:**1059–1065.
31. **Walker, S. J., P. Archer, and J. G. Banks.** 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J. Appl. Bacteriol. **68:**157–162.
32. **Wemekamp-Kamphuis, H. H., J. A. Wouters, R. D. Sleator, C. G. M. Gahan, C. Hill, and T. Abee.** 2002. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. Appl. Environ. Microbiol. **68:**4710–4716.
33. **Yamane, K., M. Kumano, and K. Kurita.** 1996. The 25 degrees-36 degrees region of the *Bacillus subtilis* chromosome: determination of the sequence of a 146 kb segment and identification of 113 genes. Microbiology **142:**3047–3056.